PATENT
Docket No. 511582002700

CERTIFICATE OF FACSIMILE TRANSMISSION

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Tami M. Procopio

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Daniel E. H. AFAR, *et al.*

Serial No.: 09/389,000

Filing Date: 31 August 1999

For: PHELIX: A TESTIS-SPECIFIC PROTEIN EXPRESSED IN CANCER

Examiner: Minh-Tam B. Davis

Group Art Unit: 1642

DECLARATION OF PIA M. CHALLITA-EID

UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Pia M. Challita-Eid, declare as follows:

1. I have a Ph.D. in Microbiology from University of Southern California, did post doctoral work at University of California at Los Angeles, and was a faculty member at the University of Rochester. I have been practicing in the field of molecular biology for over 10 years. At Agensys, I am the Group Leader of Gene Discovery. In my position at Agensys, I have responsibility for evaluating the levels of expression of various genes in tissues. A copy of my *curriculum vitae* is enclosed as Exhibit A.

2. Our company, Agensys, is dedicated to discovery of proteins that are highly expressed in various tumor tissues as compared to normal tissues. The company approaches this discovery task by first identifying cDNAs which correspond to genes overexpressed in tumor

discovery task by first identifying cDNAs which correspond to genes overexpressed in tumor tissue using the technique of suppression subtractive hybridization (SSH). In this technique, cDNA from normal tissues is subtracted from cDNA from tumor tissues. Thereby, cDNA present in tumor tissues but not in normal tissues is isolated. Thus, on a gene-by-gene basis, this approach can indicate that a gene corresponding to the cDNA is overexpressed in tumor cells.

3. Typically, the next step is to utilize the sequence information obtained from SSH to obtain a full-length DNA clone which includes the entire open reading frame for the protein corresponding to this cDNA.

4. In addition, the level of expression of the corresponding gene is determined in various normal tissues and in various tumor tissues and tumor cell lines using the technique of Northern blotting, which detects production of messenger RNA. It is well known that the production of messenger RNA, that encodes the protein, is a necessary step in the production of the protein itself. Therefore, detection of high levels of messenger RNA by, for example, Northern blot, is a way of determining that the protein itself is produced.

5. Northern blotting is a detection method of relative levels of mRNA expression of a gene. It is procedure in which specific mRNA is measured using a nucleic acid hybridization technique. The signal is detected on an autoradiogram. The stronger the signal, the more abundant is the mRNA. For genes that produce mRNA that contains an open reading frame flanked by a good Kozak translation initiation site and a stop codon, in the majority of cases the synthesized mRNA codes for a protein. Kozak translation initiation sites are discussed in greater detail paragraph 7, below.

6. The evidence referred to in paragraphs 3, 4 and 5 above is consistent with the general knowledge in the art of molecular biology that, with rare exceptions, expression of a polynucleotide is predictive of expression of the corresponding protein. This is particularly true for mRNA with an open reading frame and a Kozak consensus sequence for translation initiation.

7. The consensus Kozak initiation site CCACC*ATG*G where the ATG start codon is italicized, refers to the "optimum" translation initiation sequence. A study by Peri and Pandey *Trends in Genetics* (2001) 17: 685-687, describes a study of over 1500 translation initiation sites in order to address the natural mRNA translation initiation. This study showed that the most authentic initiation sequence has 3 or more mismatches from the optimum consensus Kozak sequence CCACCATGG. The sequence of the translation initiation site of PHELIX, TCAACATGG, shows only 2 nucleic acid differences from the optimum Kozak consensus. Also, the translation initiation site of PHELIX contains a G at position +4, which has been shown to significantly augment translation efficiency (Kozak (1997) *Embo J* 16:2482-92). Altogether, these data demonstrate that the translation initiation site of PHELIX is functional and can initiate protein translation.

8. The Northern blot technique is used as a routine procedure (as compared to Western blotting, immunoblotting or immunohistochemistry) because it does not require the time delays involved in isolating or synthesizing the protein, preparing an immunological composition of the protein, eliciting a humoral immune response, harvesting the antibodies, and verifying the specificity thereof. All of these things can be done, but they take time, and the presence of mRNA on Northern blots, especially in comparative tissues, is a recognized indication that the protein itself will be produced.

9. I am familiar with the general practice of Northern blotting and interpretation, described above, being carried out, not only at Agensys, but also at other companies that seek to evaluate gene expression in various tumor and other tissues. The use of Northern blots as a means for evaluating protein production is universally accepted as reliable and is therefore widely practiced.

10. It is understood that the absolute levels of messenger RNA present and the amounts of protein produced do not always provide a 1:1 correlation. However, in those instances where the Northern blot has shown mRNA to be present, it is almost always possible, in my experience, when the time is taken to do so, to detect the presence of the corresponding

protein in the tissue which provided a positive result in the Northern blot. The levels of the protein compared to the levels of the mRNA may be disjunctive, but is would be inaccurate to say that there is no correlation between protein levels and mRNA levels as a general matter. In general, cells that exhibit detectable mRNA also exhibit detectable corresponding protein and *vice versa*. This is particularly true where the mRNA has an open reading frame and a good Kozak sequence.

11. Ironically, studies seeking to determine the overall pattern of correlation between mRNA and corresponding protein have started with displaying the protein fingerprint of a particular cell or tissue. For instance, an article by Anderson, L. and Seilhamer, J., *Electrophoresis* (1997) 18:533-537 (Exhibit B) describes such a study on a patient liver. A 2D gel was obtained to determine the pattern of proteins in the liver, and a cDNA library was used to determine the pattern for mRNA. The authors found that of 23 selected proteins which could be identified from the gel, mRNA for 19 were detected in the transcript images. Thus, in the vast majority of cases, there was both mRNA and protein present. The authors found that the levels of RNA units to protein units had a correlation coefficient of 0.48. As they state, this number is intriguingly close to the middle position between a perfect correlation (1.0) and no correlation whatever (0.0). Only a correlation coefficient of (0.0) would support a proposition that mRNA presence provides no indication of protein presence. The conclusion has to be that in the vast majority of instances, i.e., any correlation coefficient other than (0.0), where mRNA is present protein is also present. It is inaccurate to say that there is no correlation between mRNA expression and protein expression.

12. An article by Oh, J.M.C., *et al., Proteomics* (2001) 1:1303-1319 reports a database of protein expression in lung cancer. Again, the study sought to determine the correlation between mRNA and corresponding protein beginning with protein fingerprint display of a particular cell or tissue. Protein expression was evaluated using 2D gels and mRNA expression was evaluated using microarrays. The approach is suggested as a tool for evaluating, generically, the correlation between mRNA expression and protein expression. Clearly it is expected that the correlation will not be zero or the tool would not even be proposed.

13. I am aware that the Examiner has cited a publication by Fu, L., *et al., Embo. Journal* (1996) 15:4392 - 4401 which reports an extremely rare occurrence where there does appear to be zero production of any protein even in the presence of mRNA. This is for the specific protein p53. I am not familiar with any other instances where this occurs. This is an exception to the rule that there is at least some correlation between mRNA presence and protein production. This is supported by the publication itself; were this not an unusual occurrence, this lack of correlation would not merit publication at all.

14. In many cases, a reported lack of protein expression is due to technical limitations of the protein detection assay. For instance, the available antibody may only detect denatured protein but not native protein present in a cell. In other instances, the half-life of the protein is very short, thereby the steady-state protein levels are below detectable range. Short-lived proteins are still functional, and some have been previously described to induce tumor formation as shown in the article by Reinstein *et al. Oncogene 19: 5944-50.* In such situations, when more sensitive detection techniques are performed and/or other antibodies are generated, protein expression is detected. When studies fail to take these principles into account, they are likely to report artifactually lowered correlations of mRNA to protein.

15. A previous declaration has been submitted in this case to demonstrate that, at least in 293 cells, it is possible to produce the protein encoded by the PHELIX gene. As described in Dr. Hubert's declaration, this has been verified by producing antibodies raised against a 15-mer peptide designed from the PHELIX coding region. This demonstrates that in 293 cells, there is no translational inhibition to the production of protein.

16. The production of protein in the 293 cells shows conclusively that for those tumor cells, and by analogy for tumor cell lines where mRNA is also shown to be present, the PHELIX protein is present as well. The reason I conclude this is that in this experiment, when PHELIX mRNA was made, PHELIX protein was also produced and detected. This shows that the PHELIX mRNA is stable, functional and codes for a protein. And that the

translation initiation and termination sites of PHELIX are functional sites and lead to the production of a detectable PHELIX protein.

17. Most genes, when they produce mRNA that contains an open-reading frame flanked by a good Kozak translation initiation site and a stop codon, the synthesized mRNA code for a protein. Analysis of PHELIX shows a strong mRNA signal on Northern blot in cancer tissues, and the mRNA sequence shows an open-reading frame containing a good Kozak initiation site and a stop codon. Therefore, production of PHELIX protein is reasonably predicted based on this data.

18. In summary, the scientific community regards the presence of mRNA in cells is indicative of the production of protein. This is particularly true when the Northern data is strong and the mRNA has an open reading frame and a good Kozak sequence. It is understood that the correlation of mRNA and protein levels is not perfect, however, instances such as those in Fu, where protein is absent although mRNA is present at high levels, are a rare exception.

19. The use of positive Northern blots as indicative of and predictive of protein production is a recognized conclusion of scientists in this field.

20. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at Santa Monica CA on May 10, 2002.

Pia M. Challita-Eid

Curriculum Vitae

PIA M. CHALLITA-EID, PH.D

Personal information

Work address:	Agensys, Inc. 1545 17th Street Santa Monica, CA 90404
Email:	*pchallita@agensys.com*
Home address:	15745 Morrison Street Encino, CA 91436

Appointments:

Group Leader, Research Scientist III Gene Discovery	Agensys, Inc. October 2001-Present
Research Scientist II	Agensys, Inc. August 2000-Present
Assistant Professor in Medicine, Microbiology & Immunology	University of Rochester Cancer Center Hematology/Oncology Unit July 1998- June 2000
Senior Instructor	University of Rochester Cancer Center Department of Oncology January 1996- June 1998

Education:

B.S. Biology	American University of Beirut-Lebanon 1984-1987
M.S. Microbiology	American University of Beirut-Lebanon 1987-1989

Ph.D. Microbiology	University of Southern California Department of Microbiology January 1990 - June 1994
Advisor:	Donald B. Kohn, M.D., Associate, Professor Departments of Pediatrics and Microbiology Division of Research Immunology and Bone Marrow Transplantation Childrens Hospital of Los Angeles University of Southern California, California USA
Postdoctoral fellowship	University of California Los Angeles Department of Hematology-Oncology September 1994 - December 1995
Advisor:	Joseph D. Rosenblatt, M.D., Assistant Professor School of Medicine Department of Hematology-Oncology University of California, Los Angeles, California

Students and Research Associates Mentored:

Currently leading the Gene Discovery group of 6 research associates. Previous students and research associates mentored are listed below.

1. Skelton Diane, Research Associate, 1992-1994.
2. El-Khoueiry Anthony, Undergraduate student, Summer 1992 and 1993. Currently Fellow at the USC Medical Center.
3. Poles Tina, Research Associate, 1996-1998.
4. Mosammaparast Nima, Undergraduate student, June 1996 - September 1997. Currently enrolled in Medical School.
5. Zoric Bojan, Undergraduate student, June 1997-June 1998. Currently enrolled in Medical School.
6. Rimel BJ, Research Associate, June 1998-June 1999.
7. Vicki Houseknecht, Research Associate, June 1999 – June 2000.
8. Facciponte John, Graduate student in the Microbiology and Immunology Department at the University of Rochester, January 1998 - June 2000. Currently a graduate student at Roswell Park Cancer Center, Buffalo, NY.
9. Kyung Yi, Graduate Student in Microbiology, January 1999 - June 2000.
10. Anagha Joshi, Post-doctoral fellow, October 1999 – June 2000.

Patents:

In the last year, I have been involved in the filing of greater than 40 applications.

1) "Retroviral Vectors for Expression in Embryonic Cells", US5707865, issued date Jan. 13, 1998.
2) "Chimeric Proteins for the Stimulation of a Tumor-Specific Immune Response", application in progress.

Invited Presentations:

October 1994 "Retroviral Vector Expression in Murine Stem Cells". Department of Hematology-Oncology, UCLA Gene Therapy Program, Los Angeles, California.

October 1997 "Antibody Fusion Proteins for the Specific Recruitment and Activation of an Anti-Tumor immune Response". Childrens Hospital of Los Angeles, Los Angeles, California.

February 1998 Regional Cancer Center Consortium for Biological Therapy. Roswell Park Cancer Institute, Buffalo, New York.

July 1998 American Cyanamid Company. Lederle-Praxis Biologicals Division, Rochester, New York.

October 1999 "Monoclonal Antibody Technology in the Era of Genetic Engineering" Brazilian Meeting on Biosafety and Transgenic Products, Rio De Janeiro, Brazil.

June 1999 "Breast Cancer Research in the Era of Genetic Engineering", Breast Cancer Coalition of Rochester, Rochester, NY.

Awards:

Graduate Student Research Forum Award. Silencing of retroviral vectors after transduction of hematopoietic stem cells is associated with methylation. Graduate Student Research Forum Poster Session. USC Medical School, Los Angeles, California, 1993.

Presidential Award. Society of Biological Therapy, Pasadena, California, October 1997.

Merit Award. American Society of Clinical Oncology, California, May 1998.

Grants/Funds:

1) Jonsson Cancer Center Foundation/UCLA
Fellowship Seed Grant
Title: "Antigen Processing in Human Neural Crest Tumors"

Effective Dates: 11/1/95-10/31/96
Amount: $27,707

2) Rochester Area Foundation
Lucille B. Kesel Fund for the Advancenent of Cancer Research
Title: "Antibody Fusion Proteins for Eradication of Minimal Residual Disease"
Effective Dates: 1/1/98-12/31/98
Amount: $8,000

3) University of Rochester Cancer Center
Interim and Pilot Project Funding
P.I.: Joseph D. Rosenblatt, M.D.
Co-P.I.: Pia M. Challita-Eid, Ph.D.
Title: "Antibody Fusion Proteins for the Therapy of Cancer".
Effective Dates: 1/1/98-12/31/98
Amount: $25,000

4) Sinsheimer Scholar Award
Title: "Genetically-Engineered Chemokine Antibody Fusion Proteins for Breast and Ovarian Cancer Therapy"
Effective Dates: 7/1/98-6/30/01
Amount: $40,000/year

5) NIH/NCI
P.I.: Joseph D. Rosenblatt, M.D.
Co-P.I.: Pia M. Challita-Eid, Ph.D.
Title: "Recruitment and Activation of an Anti-tumor Response using Antibody-Fusion Proteins"
Effective Dates: 12/1/98-11/30/03
Amount: $191,046/year

6) NIH/NCI - Rapid Access to Intervention Development (RAID)
Title: "Preclinical Development of a B7.1 Anti-HER2/neu Antibody Fusion Protein"
Effective Date: Approved April, 1999
Amount: Not applicable

7) ACS Institutional grant
Title: "Chemokine Directed Targeting of Cytotoxic TALL-104 Cells"
Effective Dates: 9/1/99-8/30/00
Amount: $8,000

8) Breast Cancer Coalition of Rochester
Title: "Breast Cancer Research"
Date: 9/99
Amount: $1,000

Publications:

Gersuk GM, Westermark B, Mohabeer AJ, **Challita PM**, Pattamakom S, and Pattengale, PK. Inhibition of human natural killer cell activity by platelet-derived growth factor (PDGF). III. Membrane binding studies and differential biological effects of recombinant PDGF isoforms. *Scand J Immunol* 33: 521-532, 1991.

Gersuk GM, Carmel R, **Challita PM**, Rabinowitz AP, and Pattengale PK. Quantitative and functional studies of impaired natural killer (NK) cells in patients with myelofibrosis, essential thrombocytopenis, and polycythemia vera. I. A potential role for platelet-derived growth factor in defective NK cytotoxicity. *Nat Immun* 12: 136-151, 1993.

Challita PM, and Kohn DB. Lack of expression from a retroviral vector in murine hematopoietic stem cells is associated with methylation *in vivo*. *Proc Natl Acad Sci (USA)* 91: 2567-2571, 1994.

Krall W, **Challita PM**, Perlmutter L, Skelton D, and Kohn DB. Cells expressing human glucocerebrosidase from a retroviral vector repopulate macrophages and central nervous system microglia after murine bone marrow transplantation. *Blood* 83: 2737-2748, 1994.

Challita PM, Skelton D, Yu XJ, El-Khoueiry A, Yu X-J, Weinberg KI, and Kohn DB. Multiple modifications in *cis* elements of the long terminal repeat of retroviral vectors leads to increased expression and decreased DNA methylation in embryonic carcinoma cells. *J Virol* 69: 748, 1995.

Ucar K, Seeger RC, **Challita PM**,Watanabe CT, Yen TL, Morgan JP, Amado R, Chou E, McCallister T, Barber JR, Jolly DJ, Reynolds P, Gangavalli R, and Rosenblatt JD. Sustained cytokine production and immunophenotypic changes in human neuroblastoma cell lines transduced with a human gamma interferon vector. *Cancer Gene Therapy* 2: 171, 1995.

Lu Y, Planelles V, Palaniappan C, Li X, **Challita-Eid PM**, Amado R, Stephens D, Kohn DB, Bakker A, Day B, Bambara RA, and Rosenblatt JD. Inhibition of HIV-1 replication using a mutated tRNALys3 primer. *J Biol Chem* 272: 14523, 1997.

Challita-Eid PM, Penichet ML, Shin SU, Poles T, Mosammaparast N, Mahmood K, Slamon DJ, Morrison SL, and Rosenblatt JD. A B7.1-antibody fusion protein retains antibody specificity and ability to activate*via* the T cell costimulatory pathway. *J Immunol* 160: 3419-3426, 1998.

Challita-Eid PM, Abboud CN, Morrison SL, Penichet ML, Rosell KE, Poles T, Hilchey SP, Planelles V, and Rosenblatt JD. A RANTES- antibody fusion protein retains antigen specificity and chemokine function. *J Immunology* 161: 3729, 1998.

Challita-Eid PM, Rosenblatt JD, Day B, Rimel BJ and Planelles V. Inhibition of HIV-1 infection with a RANTES.IgG3 fusion protein. *AIDS Research and Human Retroviruses* 14:1617, 1998.

Mahmood K, Federoff HJ, **Challita-Eid PM**, Day B, Haltman M, Atkinson M, Planelles V, and Rosenblatt JD. Eradication of pre-established lymphoma using HSV amplicon vectors. *Blood* 93: 643, 1999

Penichet ML, **Challita PM**, Shin S-U, Sampogna S, Rosenblatt JD, and Morrison SL. In vivo properties of three human HER2/neu-expressing murine cell lines in immunocompetent mice. *Laboratory Animal Science* 49: 179-88, 1999.

Penichet ML, Dela Cruz JS, **Challita-Eid PM**, Rosenblatt JD, Morrison SL. A murine B cell lymphoma expressing human HER2/neu undergoes spontaneous tumor regression and elicits antitumor immunity. *Cancer Immunol Immunother* 49:649-62, 2001.

Hilchey SP, Rosebrough SF, Morrison SL, Rosenblatt JD, and **Challita-Eid PM**. Specific targeting and stimulation of in vivo anti-tumor response using a B7.1 T-cell costimulatory antibody fusion protein. *Manuscript in preparation.*

Select Abstracts and Presentations:

Challita PM, El-Khoueiry AB, and Kohn DB. Silencing of retroviral vectors after transduction of murine hematopoietic stem cells is associated with methylation. *Blood* 80 (10 Suppl. 1): 168a, 1992.

Challita PM, Cook C, Sender LS, and Kohn DB. Novel retroviral vectors for consistent expression after transduction into hematopoietic stem cells. Keystone Symposium on Gene Therapy, Keystone, Colorado, 1993.

Challita PM. Retroviral vector expression in murine stem cells. Presentation. Division of Hematology-Oncology, University of California Los Angeles, October, 1994.

Challita PM, Shin S-U, Penichet M, Mahmood K, Poles TM, Rosell KE, Abboud CN, Morrison SL, Rosenblatt JD. Novel Antibody Fusion Proteins for the Stimulation of a Tumor-Specific Immune Response. Keystone Symposium on Cellular Immunology and Immunotherapy of Cancer, Copper Mountain, Colorado, January 1997.

Penichet ML, **Challita PM**, Shin S-U, Slamon DJ, Rosenblatt JD, and Morrison SL. In vivo properties of two human her2/neu expressing murine cell lines in immunocompetent mice. Mutlidisciplinary Approaches to Cancer Immunotherapy, Bethesda, Maryland, June 1997.

Challita PM, Abboud CN, Rosell KE, Penichet ML, Poles T, Mahmood K, Morrison SL, and Rosenblatt JD. Characterization of a RANTES-antibody fusion protein for cancer immunotherapy. Mutlidisciplinary Approaches to Cancer Immunotherapy, Bethesda, Maryland, June 1997.

Horwitz S, Rosenblatt JD, Mosammaparast N, Poles T, Abboud CN, and **Challita PM**. Gene-modified EL4 cells expressing the chemokine RANTES protects from tumor growth and stimulates an anti-tumor cytotoxic T-lymphocyte response *in vivo*. Mutlidisciplinary Approaches to Cancer Immunotherapy, Bethesda, Maryland, June 1997.

Challita-Eid PM, Morrison SL, Penichet ML, Rosenblatt JD. Antibody-T cell costimulatory ligand fusion protein for the stimulation of a specific anti-tumor immune response. American Society of Hematology, San Diego, California, December 1997.

Challita-Eid PM, Abboud CN, Penichet ML, Rosell KE, Morrison SL, Rosenblatt JD. Antibody fusion proteins for the recruitment and activation of an anti-tumor immune response. American Association for Cancer Research. New Orleans, Louisiana, March 1998.

Challita-Eid PM, Hilchey Shannon P., and Rosenblatt Joseph D. An anti-HER2/neu RANTES fusion protein induces effector cell infiltration to the site of HER2/neu expressing tumors. AACR/NCI/EORTC Molecular Targets and Cancer Therapeutics, Washington DC, November 1999.

Facciponte JG, Rosenblatt JD, H.J.Federoff HJ, **Challita-Eid PM**. Herpes simplex virus (HSV) amplicon-mediated gene transfer of tumor associated antigens into bone marrow derived dendritic cells. Keystone Symposium on Cellular Immunity and Immunotherapy of Cancer, Santa Fe, New Mexico, January 2000.